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Bigendothelin-1 via p38-MAPK-dependent mechanism regulates adult rat ventricular myocyte contractility in sepsis

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Abstract

We tested the hypothesis that exogenous administration of the ET-1 precursor, bigET-1, would regulate adult rat ventricular myocyte (ARVM) contractility in a p38-mitogen activated protein kinase (p38-MAPK)-dependent mechanism during sepsis. Ventricular myocytes from adult rat hearts (both sham and septic) were stimulated to contract at 0.5 Hz and mechanical properties were evaluated using an IonOptix Myocam system. Immunoblot analysis was used to determine the phosphorylation of p38-MAPK and extracellular signal-regulated kinase 1/2 (ERK1/2). ARVMs were treated with vehicle, bigET-1 and inhibitors for 24 h and then subjected to functional and biochemical estimations. Septic ARVM displayed a distorted cell membrane and irregular network within the cells along with increased cell contractility as evidenced by elevated peak shortening (PS), maximal velocity of shortening (+dL/dt) and relengthening (−dL/dt) in comparison to sham ARVM. BigET-1 treatment caused ARVM enlargement in both sham and sepsis groups. BigET-1 (100 nM) produced an increase in ARVM contractility in sham group as compared to vehicle treatment. However, septic ARVM treated with bigET-1 exhibited unaltered ARVM contractility, and upregulated ET_B receptors as compared to respective sham group. BigET-1 increased the concentration of ET-1 and upregulated phosphorylation of p38-MAPK but not of ERK1/2 in sham and septic ARVM. Furthermore, inhibition of p38-MAPK by SB203580 (10 μM) increased ARVM contractility in sham but not in sepsis group. BigET-1 reversed SB203580-induced increase in PS in sham group but accentuated it in sepsis group. BigET-1 also reversed SB203580-induced inhibition of p38-MAPK phosphorylation in sham but not in septic ARVM. SB203580 pretreatment followed by bigET-1 administration significantly decreased p38-MAPK phosphorylation and downregulated ET_B receptor expression as compared to bigET-1 treatment per se in sepsis group but not in sham. We concluded that a bigET-1-induced non-responsive effect on septic ARVM contractile function could be due to upregulation of p38-MAPK phosphorylation and ET_B receptor expression.

Keywords: p38 -MAPK; ERK1/2; Endothelin-1; Adult rat ventricular myocyte; Peak shortening; Immunoblot analysis

Abbreviations: ANOVA, analysis of variance; ARVM, Adult Rat Ventricular Myocytes; bigET-1, big endothelin-1; CHF, chronic heart failure; CLP, cecal ligation and puncture; D_5W, 5% dextrose water; +dL/dt, maximal velocity of myocyte shortening; −dL/dt, maximal velocity of myocyte relengthning; ECE-1, endothelin converting enzyme- 1; ERK1/2, extracellular signal-regulated kinase 1 and 2; ET-1, endothelin-1; IL-10, interleukin-10; i.p., intraperitoneal; JNK, c-jun N-terminal kinase; KHB, Krebs–Henseleit's buffer; LPS, lipopolysaccharide; M199, Medium 199; MAPK, mitogen activated protein kinases; NO, nitric oxide; NOS2, iNOS, inducible nitric oxide synthase, nitric oxide synthase II; PS, peak shortening; pp38-MAPK, phosphorylated p38-MAPK; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; SIRS, systemic inflammatory response syndrome; SHR, spontaneously hypertensive rats; TBST, tris buffer saline with tween20

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1. Introduction

Sepsis is a complex progressive immunological, metabolic and cardiovascular disorder that results from dysregulation of normally protective anti-microbial host defense mechanisms followed by the development of Systemic Inflammatory Response Syndrome (SIRS) in patients [1]. Several studies have indicated myocardial injury characterized by hemodynamic alterations including hypotension and depressed myocardial contractility during sepsis [2]. In our chronic peritoneal sepsis rat model, we have demonstrated depressed left ventricular velocity of contraction and relaxation in an isolated heart preparation [3] and reduced rate of myocyte shortening in isolated adult rat ventricular myocytes (ARVM) [4]. However, the mechanism underlying ARVM contractile dysfunction during sepsis has not yet been elucidated.

Numerous reports suggest modulation of vasoactive mediators like endothelin-1 (ET-1) during sepsis in patients and animal models [3,5]. ET-1 is not only a potent vasoconstrictor but also a mitogenic and hypertrophic factor [6,7]. ET-1 is synthesized following activation of preproET-1 mRNA and subsequently processed by proteolytic enzymes to generate a propeptide bigET-1. Endothelin converting enzyme (ECE-1) then cleaves bigET-1 to a biologically active 21 amino acid peptide that is 140 times more potent than bigET-1 [6,8]. ET-1 is expressed in cardiac myocytes [9] and can affect contractile properties, inducing cardiac hypertrophy and cellular injury of cardiac myocytes [7,10]. Earlier, we have demonstrated elevated concentration of myocardial ET-1 at 4, 8 and 12-h post-sepsis [3]. Therefore, in the present study, we determined if treatment of ARVM with ET-1 precursor, bigET-1 for 24 h would affect the contractile functional properties of septic ARVM.

Stress, SIRS and ET-1 stimulate manifold cellular signaling molecules including mitogen-activated protein kinases (MAPKs) [11–13]. MAPKs are a superfamily of serine–threonine protein kinases that mediate a multitude of cellular events such as cell survival, cell injury, cell proliferation or differentiation and apoptosis [14]. Three MAPK subfamilies have been well characterized: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-jun N-terminal kinase (JNK) and p38-MAPK (13). ERKs are implicated in the regulation of growth response while p38-MAPK and JNK regulate cellular response to stress. Myocardial p38-MAPK activation has been observed in pressure overload, ischemia, cardiac hypertrophy and heart failure in humans [15] and animal models [16]. We have also observed increased phosphorylation of myocardial p38-MAPK at 24-h post-sepsis [17]. Activation of p38-MAPK has been shown to attenuate cardiac contractility, in vivo, in transgenic mice [18]. Conversely, ET-1 exerts a positive inotropic effect in ARVM [19]. In our chronic peritoneal sepsis model, both ET-1 [3] and p38-MAPK [17] are elevated along with a substantial deficit in cardiac function [3,4]. Earlier data from our laboratory indicated that increasing the substrate availability of ET-1 using bigET-1 in vivo produces left ventricular dysfunction during sepsis [20]. In addition, we have also demonstrated that ECE-1 inhibition at the time of LPS administration attenuated induction of myocardial inducible nitric oxide synthase (iNOS) and p38-MAPK phosphorylation [21]. These data led us to speculate that increase in the concentration of bigET-1 during early sepsis could play a role in sepsis-induced myocardial dysfunction that occurs later (post 24-h sepsis). However, the mechanism underlying the role of p38-MAPK and ET-1 on ARVM contractile dysfunction during sepsis is not yet known. Therefore, we hypothesized that exogenous administration of the ET-1 precursor, bigET-1, would regulate ARVM contractility in a p38-MAPK-dependent mechanism during sepsis.

2. Materials and methods

Male Sprague–Dawley rats (350–400 g) obtained from Harlan, IN were acclimatized to the laboratory conditions for at least 7 days following their arrival. All experiments were conducted in compliance with humane animal care standards outlined in the *NIH Guide for the Care and Use of Experimental Animals* and were approved by the Institutional Animal Care and Use Committee of North Dakota State University.

2.1. Induction of sepsis

Sepsis was induced in the animals using cecal inoculum (200 mg/kg, i.p.) as previously described [3]. Briefly, cecal inoculum was prepared by mixing cecal contents (200 mg) obtained from donor rats (euthanized with pentobarbital; 100 mg/kg, i.p.) with 5 ml of 5% dextrose water (D_5W) to yield a concentration of 40 mg/ml. The cecal inoculum was prepared fresh each day and cecal material from one donor rat was used within 2 h for 3–5 experimental animals.

2.2. Isolation of ARVMs

Single ARVMs were isolated from sham and septic rat hearts by cardiac retrograde aortic perfusion as described previously [4]. Briefly, heart was rapidly removed and perfused (at 37 °C) with oxygenated (5% CO_2–95% O_2) Krebs–Henseleit bicarbonate (KHB) buffer (mM: NaCl 118, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, N-[2-hydro-ethyl]-piperazine-N′-[2-ethanesulfonic acid] (HEPES) 10, glucose 11.1, pH 7.4). Heart was then subsequently perfused with a nominally Ca^{2+}-free KHB buffer for 2–3 min followed by a 20-min perfusion with Ca^{2+}-free KHB containing 223 U/ml type II collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA) and 0.1 mg/ml hyaluronidase (Sigma Chemical, St. Louis, MO, USA). After perfusion, the left ventricle was

removed, minced and further digested with trypsin (Sigma) and deoxyribonuclease before being filtered through a nylon mesh (300 μm) and subsequently separated from the enzymatic solution by centrifugation (60 × *g*, 30 s). Cells were initially washed with Ca^{2+}-free KHB buffer to remove remnant enzyme and extracellular Ca^{2+} was added incrementally back to 1.25 mM. Isolated ARVM were maintained in Medium 199 (M199) supplemented with L-carnitine (2 mM), taurine (5 mM) and penicillin–streptomycin (100 IU/ml). Preparations were considered satisfactory only if the yield of rod-shaped cells was more than 70%. The cells were not used if they had any obvious sarcolemmal blebs or spontaneous contractions. In addition, cell viability in different groups was assessed using standard MTT assay method following 24-h post-treatment using different interventions.

2.3. ARVM shortening and relengthening

Mechanical properties of ARVM were assessed by an IonOptix Myocam system (IonOptix Inc., Milton, MA, USA). Cells were placed in a chamber mounted on the stage of an inverted microscope and superfused (at 25 °C) with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES at pH 7.4. The cells were field stimulated at a frequency of 0.5 Hz and 3-ms duration. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS), maximal velocities of shortening (+dL/dt) and relengthening (−dL/dt). To test the effect of bigET-1 on ARVM contractile function, cell contractility was recorded before and 24 h after bigET-1 application to the culture medium. In some experiments, the inhibitors (SB203580, and PD98059) were present prior to and during bigET-1 application to ARVM. The inhibitors were added 15 min prior to bigET-1 treatment in the culture medium.

2.4. Determination of concentration of ET-1 in ARVM supernatants

The concentration of ET-1 was determined in ARVM supernatants using a commercially available EIA kit (Assay Designs, CA). The ARVM supernatants were acidified adding an equal volume of 20% acetic acid. The smaller peptides were extracted from ARVM supernatants using SEP-columns (21, 22) and then assayed for ET-1. The ET-1 assay has <0.1% cross reactivity with related peptides like bigET-1. Protein concentration in ARVM supernatants was determined by Bio-Rad Bradford assay. The ET-1 concentration in ARVM supernatants was expressed as pg/g protein.

2.5. Cell treatment and lysis

Freshly isolated ARVMs were incubated at 37 °C in culture medium (M199) containing vehicle, agonist or inhibitors for 24 h. ARVMs were then lysed in ice-cold buffer containing 20 mM HEPES, 20 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 10 mM benzamidine, 200 μM leupeptin, 300 μM PMSF and 1% (v/v) TritonX-100, and extracted on ice for 30 min. Cell lysates were centrifuged (10 000 × *g*, 5 min at 4 °C) and the supernatants were boiled with 0.33 vol. of sample buffer (0.33 M Tris–HCl, pH 6.8, 10% w/v SDS, 13% (v/v) glycerol, 133 mM DTT and 0.2% Bromophenol Blue). Protein concentrations were determined using Bio-Rad Bradford assay.

2.6. Immunoblot analysis

Proteins (20 μg) were electrophoresed on 10% denaturing sodium dodecyl sulfate (SDS) polyacrylamide gels. The proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane (Gelman Sciences, Pierce, Rockford, IL). Non-specific binding sites on the membrane were blocked with 5% nonfat dry milk in Tris–HCl containing 0.2% tween20 buffer (TBST) overnight at 4 °C. The membranes were then exposed to primary

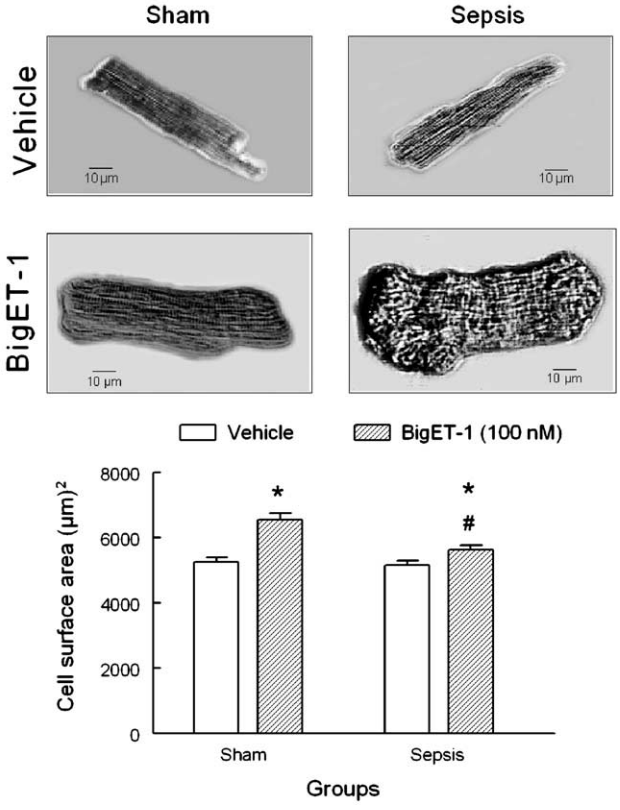

Fig. 1. (A) Representative images of ARVM treated with vehicle and bigET-1 (100 nM) in sham and sepsis groups. Treatments were done for 24 h. ARVMs were then observed under light phase contrast microscopy. Magnification=40×. (B) Effect of bigET-1 on cell cross-section area (surface area) of ARVM in sham and sepsis groups. Data are represented as mean ± S.E.M., n=50 per data group, $^{*}P \leq 0.05$ as compared to vehicle treatment, $^{\#}P \leq 0.05$ as compared to respective treatment in the sham group.

antibody for 1 h at room temperature. After five washings in TBST the membranes were incubated with the secondary antibody for 1 h at room temperature. Finally, membranes were washed three times with TBST. The specific proteins were detected by enhanced chemiluminescence (ECL reagent, Amersham Pharmacia Biotech). The blots were analyzed using Un-Scan-It software to estimate the density of the blots in pixels. Uniform loading was assessed by β-actin protein expression [21,23].

2.7. Pharmacological interventions and antibodies

BigET-1 (1–38) was obtained from American Peptide, Sunnyvale, CA, in sterile normal saline to make a stock solution (1 mg/ml). Since bigET-1 is ~140 times less potent than mature ET-1 (6,8), we used bigET-1 at the concentration of 10, 50 and 100 nM that would produce a response equivalent to approximately 0.1 to 1 nM of mature ET-1 in the supernatant. SB203580 and PD98059 were obtained from Calbiochem, La Jolla, CA and were prepared as 10 mM and 25 mM stock solutions, respectively, in DMSO. The required amount of intervention from the stock solution was added in 5 ml of M199 to get the desired concentration. Mouse monoclonal antibodies to phosphorylated p38-MAPK, ERK1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to total p38-MAPK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), total ERK1/2 from Sigma (St. Louis, MI), ET_A and ET_B receptors from Abcam Inc. (Cambridge, MA).

2.8. Statistical analyses

Data are presented as mean ± S.E. The functional and biochemical data were analyzed by one-way ANOVA using *SPSS* software. After obtaining a significant F value, post hoc Student–Newman–Keul's test was performed for inter- and intra-group comparisons. Statistical significance was realized at $P \leq 0.05$ to approve the null hypothesis for individual parameters.

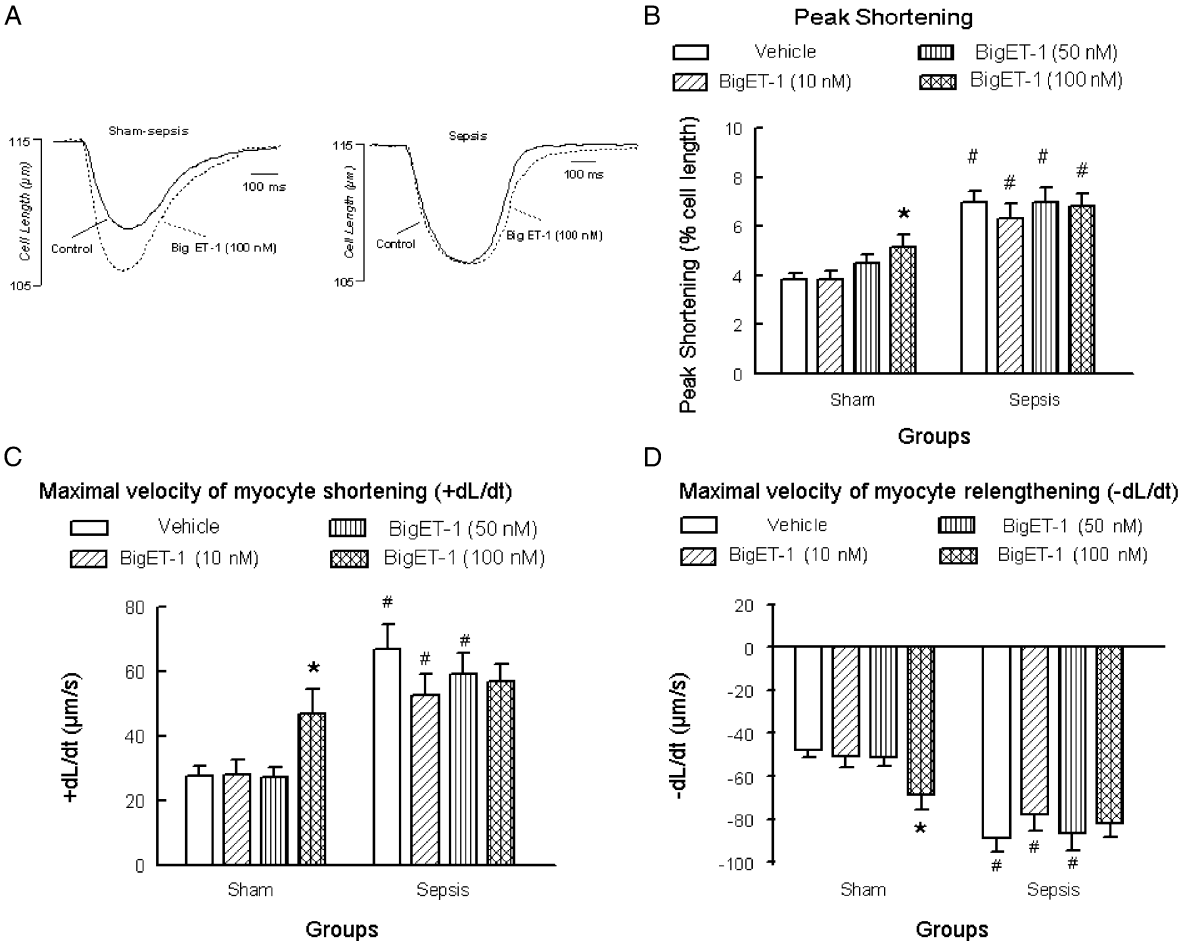

Fig. 2. Representative traces depicting the effect of bigET-1 on cell shortening in ARVM in sham (A1) and sepsis (A2) groups. Effect of bigET-1 (10, 50 and 100 nM) on (B) peak shortening, (C) maximal velocity of myocyte shortening (+dL/dt), (D) maximal velocity of myocyte relengthening (−dL/dt) in sham and sepsis groups. Data are represented as mean ± S.E.M., n=50 per data group, $^{*}P \leq 0.05$ as compared to vehicle treatment, $^{\#}P \leq 0.05$ as compared to respective treatment in the sham group.

3. Results

3.1. General characteristics of animals and ARVMs

The septic animals uniformly displayed piloerection, lethargy, periocular discharge and diarrhea. Post-mortem analysis of the abdominal cavity of septic rats revealed the presence of ascites and infarcts on peritoneal organs including gastrointestinal tract, liver and kidney. The peritoneal cavity was profusely ulcerated with an extremely foul smelling peritoneal fluid. In contrast, sham rats were freely moving after treatment with D_5W and did not exhibit presence of ascites or infarcts on post-mortem analysis.

ARVMs of septic animals displayed distorted cell membrane and irregular network within the cells (Fig. 1). The cell length observed immediately after isolation (at 0 h treatment) was 100 ± 10 μm and 115 ± 10 μm in sham and septic ARVM, respectively. However, post 24 h, the cell lengths were 142 ± 4 μm and 144 ± 4 μm in sham and sepsis groups. BigET-1 treatment produced enlarged ARVMs in both sham and sepsis groups (Fig. 1). The percent viability of ARVM isolated from septic animals was 92% as compared to 100% of sham group. BigET-1 depressed the viability of ARVMs both in sham (74%) and septic (46%) groups.

3.2. Effect of bigET-1 on ARVM contractility

Representative changes in the cell length of an individual ARVM following treatment with bigET-1 (100 nM) in sham and sepsis group are shown in Figs. 2—A1 and A2, respectively. Sham ARVM displayed an increase in con-

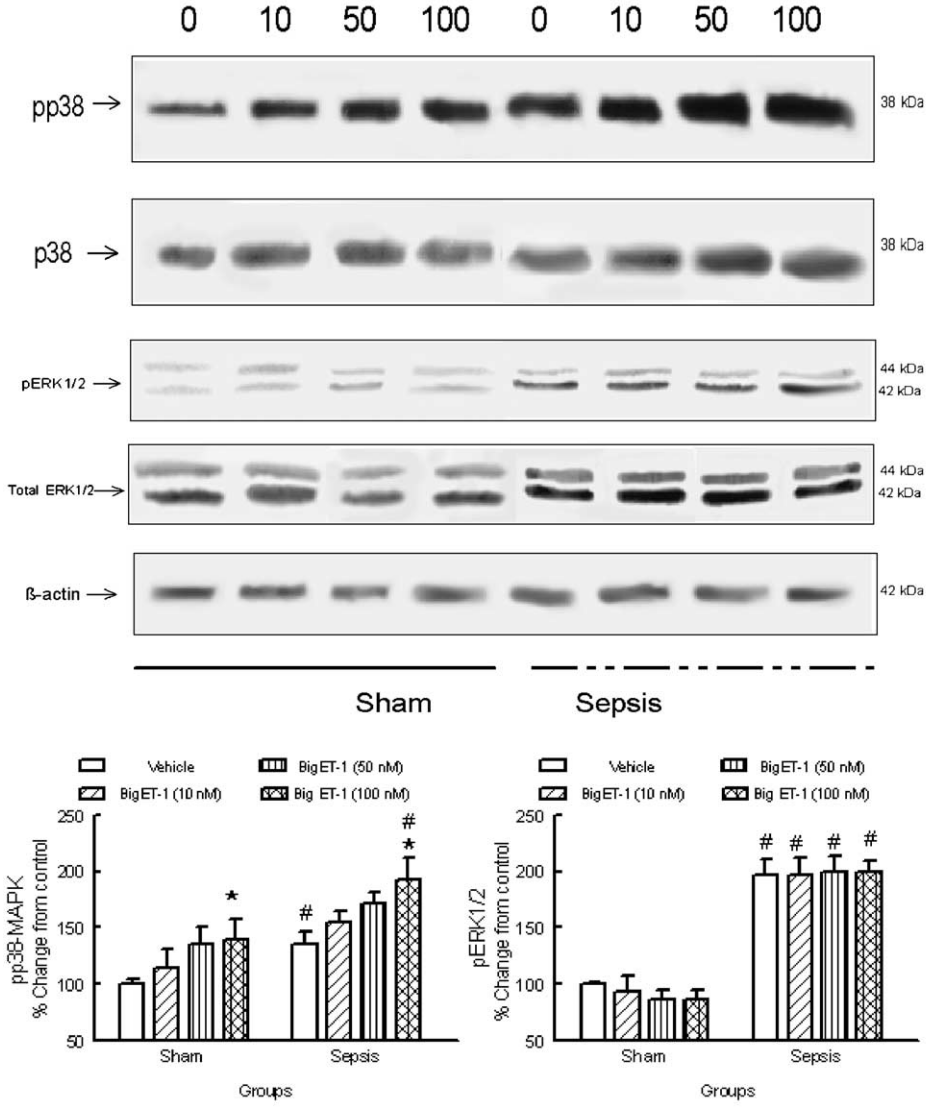

Fig. 3. Effect of bigET-1 (10, 50, and 100 nM) on the phosphorylation of p38-MAPK and ERK1/2 in ARVM obtained from sham and sepsis groups. Cell lysates were also immunobloted for total p38-MAPK and ERK1/2 (lower panel). The blot in each case is a representative of three independent experiments. β-actin protein expression depicts uniform loading in gel. Data are represented as mean ± S.E.M., $^{*}P \leq 0.05$ as compared to vehicle treatment, $^{\#}P \leq 0.05$ as compared to respective treatment in the sham group.

traction amplitude over time following treatment with bigET-1 (Fig. 2A1) which was not seen in sepsis group (Fig. 2A2). BigET-1 (100 nM, 24 h treatment) produced a significant increase (34%) in PS as compared to vehicle treatment in sham group (Fig. 2B). BigET-1 also significantly increased +dL/dt (Fig. 2C) and −dL/dt (Fig. 2D) as compared to vehicle treatment in sham ARVM. Septic ARVM exhibited elevated PS, +dL/dt and −dL/dt as compared to sham group. However, no significant differences in PS, +dL/dt and −dL/dt were observed following bigET-1 treatment in the sepsis group (Fig. 2).

In a separate series of experiments it was observed that in sham ARVM bigET-1 caused a 31% and 41% increase in peak shortening (PS) at 3 h and 6 h as compared to the baseline values. There were no significant changes in ±dL/dt at either of the time-points. Septic ARVM exhibited a depressed contractile function at 0 h (33%) as compared to sham. BigET-1 treatment produced an increase in ±dL/dt and PS at 3 h as compared to 0 h in the sepsis group. No significant differences in the ±dL/dt and PS values were observed at 6 h post-bigET-1 treatment, as compared to the respective vehicle-treated sham and septic groups.

3.3. Effect of bigET-1 on p38-MAPK and ERK1/2 phosphorylation

To determine bigET-1 induced activation of p38-MAPK and ERK1/2, protein expression of phosphorylated p38-MAPK and ERK1/2 was examined. Twenty-four hours incubation of septic ARVM in culture exhibited an upregulation of p38-MAPK and ERK1/2 phosphorylation as compared to sham group. BigET-1 (100 nM) significantly stimulated the phosphorylation of p38-MAPK in both sham and sepsis groups. However, bigET-1 had no effect on ERK1/2 phosphorylation in either group at any of the doses studied (Fig. 3).

Since ET-1 can cause an increase in p38-MAPK phosphorylation at earlier time points, in a separate series of experiments, the effect of bigET-1 (100 nM) was observed at 5, 15 and 30 min on p38-MAPK phosphorylation in sham and septic ARVMs. Septic ARVM exhibited a significant progressive increase in the phosphorylation of p38-MAPK from 5 to 30 min following bigET-1 administration. This increase was more than 5-fold as compared to respective sham values at 30 min. Sham ARVM did not show any significant changes in the expression of phosphorylated p38-MAPK at any of the time-points. The expression of total p38-MAPK was not significantly different in any of the groups. Phosphorylated ERK1/2 expression was significantly increased in the septic ARVM at 5, 15 and 30 min as compared to the respective sham groups. However, there were no significant changes in p-ERK1/2 expression in the sham ARVM. Total ERK1/2 expression was not significantly altered in the sham or septic ARVM (Data not shown).

3.4. Effect of bigET-1 on the concentration of mature ET-1

BigET-1 (10, 50 and 100 nM) produced a significant increase in ET-1 levels as compared to vehicle treatment in both sham and sepsis groups (Fig. 4).

3.5. Effect of SB203580 on ARVM viability and contractility during sepsis

We investigated the effect of p38-MAPK on the contractile function of ARVM during sepsis by inhibiting p38-MAPK activity using a synthetic p38-MAPK inhibitor, SB203580. Sham and septic ARVM following treatment with SB203580 were 99% and 96% viable at 24 h post-treatment. Treatment of ARVM with SB203580 (1 and 10 μM) for 24 h significantly increased PS by approximately 2-fold in sham group as compared to vehicle treatment (Table 1). SB203580 at both the doses produced a significant increase in +dL/dt and −dL/dt as compared to vehicle treatment in sham group (Table 1). The effect of SB203580 (10 μM) on +dL/dt and −dL/dt was more pronounced than SB203580 (1 μM) in sham ARVM. Hence, we have used SB203580 (10 μM) for further experiments in the current study. Although SB203580 at both the concentrations in septic ARVM did not affect PS, it produced a significant reduction in PS, +dL/dt and −dL/dt as compared to the respective treatments in sham group (Table 1).

BigET-1 activated phosphorylation of p38-MAPK (Fig. 3) and depressed cell viability (74% in sham and 46% in sepsis group). SB203580 (10 μM) attenuated bigET-1-induced decrease in cell viability and increased it to 85% in sham group and 71% in sepsis group. To examine the involvement of p38-MAPK on bigET-1-induced ARVM contractility alterations, the effect of bigET-1 on PS, +dL/dt and −dL/dt was reexamined in presence of SB203580 (10 μM). SB203580 pretreatment followed by bigET-1 (100 nM) administration in sham group did not alter bigET-1

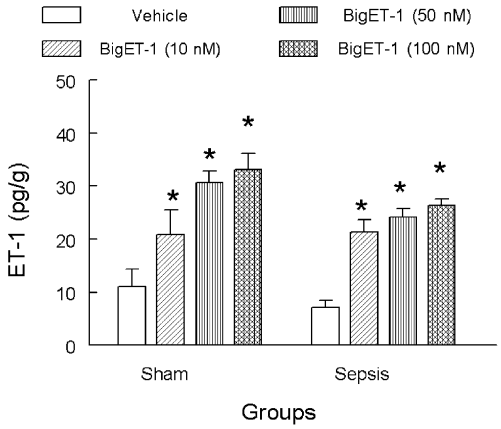

Fig. 4. Effect of bigET-1 (10, 50, and 100 nM) on the concentration of ET-1 (pg/g protein) in the supernatants of ARVM obtained from sham and sepsis groups. Data are represented as mean ± S.E.M., *$P \leq 0.05$ as compared to vehicle treatment.

Table 1
Effect of SB203580 and PD98059 on peak shortening (PS) and maximal velocities of myocyte shortening (+dL/dt) and relengthening (−dL/dt)

Groups	PS		+dL/dt		−dL/dt	
	Sham	Sepsis	Sham	Sepsis	Sham	Sepsis
Vehicle	3.9±0.4	7.0±0.5*	27.8±2.8	67.0±7.6*	−47.9±3.2	−89.1±6.2*
SB203580 (1 μM)	8.0±0.5**	6.6±0.5*	92.3±9.7**	72.0±8.7*	−116.2±8.3**	−94.3±7.3*
SB203580 (10 μM)	7.9±0.6**	6.0±0.5*	114.5±14.2**,***	59.0±6.7*	−136.7±9.8**,***	−84.2±6.7*
PD98059 (10 μM)	6.1±0.4**	6.0±0.5	63.2±6.5**	61.7±7.8	−84.9±5.6**	−88.8±6.1
PD98059 (50 μM)	5.3±0.4**	5.8±0.4	51.9±5.5**	64.8±7.3	−79.4±6.3**	−87.2±6.6

Data represent mean ± S.E.M., n=50 cells.
* $P \leq 0.05$ as compared to respective treatment in the sham group.
** $P \leq 0.05$ as compared to vehicle treatment.
*** $P \leq 0.05$ as compared to SB203580 (1 μM) treatment in the respective group.

induced increase in PS (Fig. 5A), +dL/dt and −dL/dt (Figs. 6A1, A2). However, SB203580 pretreatment caused a significant depression on PS, +dL/dt and −dL/dt as compared to SB203580 treatment per se in sham group. Septic ARVM on pretreatment with SB203580 followed by bigET-1 administration produced a significant increase in PS, +dL/dt and −dL/dt as compared to bigET-1 and SB203580 treatments per se. This effect of SB203580 pretreatment in sepsis group was significantly different than the sham group (Figs. 5 and 6).

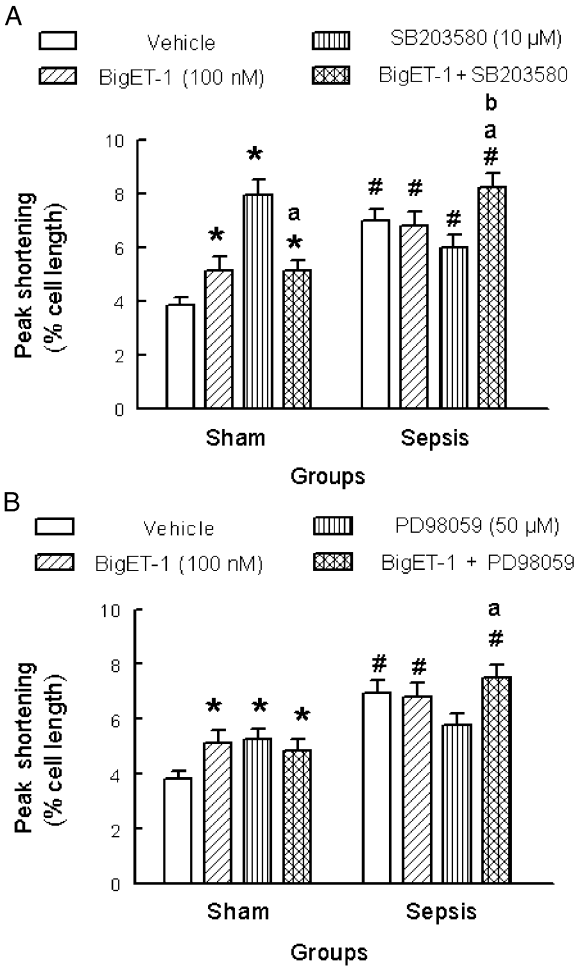

Fig. 5. Effect of inhibitors (A) SB203580 (10 μM) and (B) PD98059 (50 μM) on peak shortening in ARVM obtained from sham and sepsis groups in presence and absence of bigET-1 (100 nM). Data are represented as mean ± S.E., n=50 per data group, *$P \leq 0.05$ as compared to vehicle treatment, #$P \leq 0.05$ as compared to respective treatment in the sham group, a$P \leq 0.05$ as compared to inhibitor treatment in the respective group, b$P \leq 0.05$ as compared to bigET-1 (100 nM) treatment in the respective group.

3.6. Effect of PD98059 on ARVM viability and contractility during sepsis

PD98059 depressed the cell viability in sham (40%) and septic (24%) group and remained depressed following treatment with bigET-1 in sham (38%) and septic (24%) group. We assessed the effect of ERK1/2 inhibitor PD98059 on ARVM contractility during sepsis. A significant increase in PS, +dL/dt and −dL/dt was observed by PD98059 treatment at both 10 μM and 50 μM in sham group as compared to vehicle treatment. However, PD98059 did not affect the ARVM contractile function (PS, +dL/dt and −dL/dt) of ARVM in sepsis group at either dose as compared to vehicle treated septic ARVM and respective treatments in sham group (Table 1).

Pretreatment with PD98059 (50 μM) followed by bigET-1 treatment significantly increased PS, +dL/dt and −dL/dt as compared to vehicle treatment in sham group. However, this effect was not significantly different as compared to the respective treatments per se in sham group (Figs. 5B and 6B1, B2). Sepsis produced a significant increase on PS, +dL/dt and −dL/dt (Figs. 6B1, B2) on PD98059 pretreatment followed by bigET-1 administration as compared to the respective sham group. During sepsis, +dL/dt but not PS and −dL/dt in the PD98059 pretreatment group was significantly different as compared to bigET-1 treatment. Also, the PS following PD98059 pretreatment in the sepsis group was significantly increased as compared to PD98059 treatment per se (Figs. 5 and 6).

3.7. Effect of bigET-1 on p38-MAPK phosphorylation in presence of SB203580 and PD98059

SB203580 (10 μM) produced a significant decrease in p38-MAPK phosphorylation as compared to vehicle treatment in sham group. SB203580 did not alter p38-MAPK phosphorylation in sepsis group as compared to vehicle

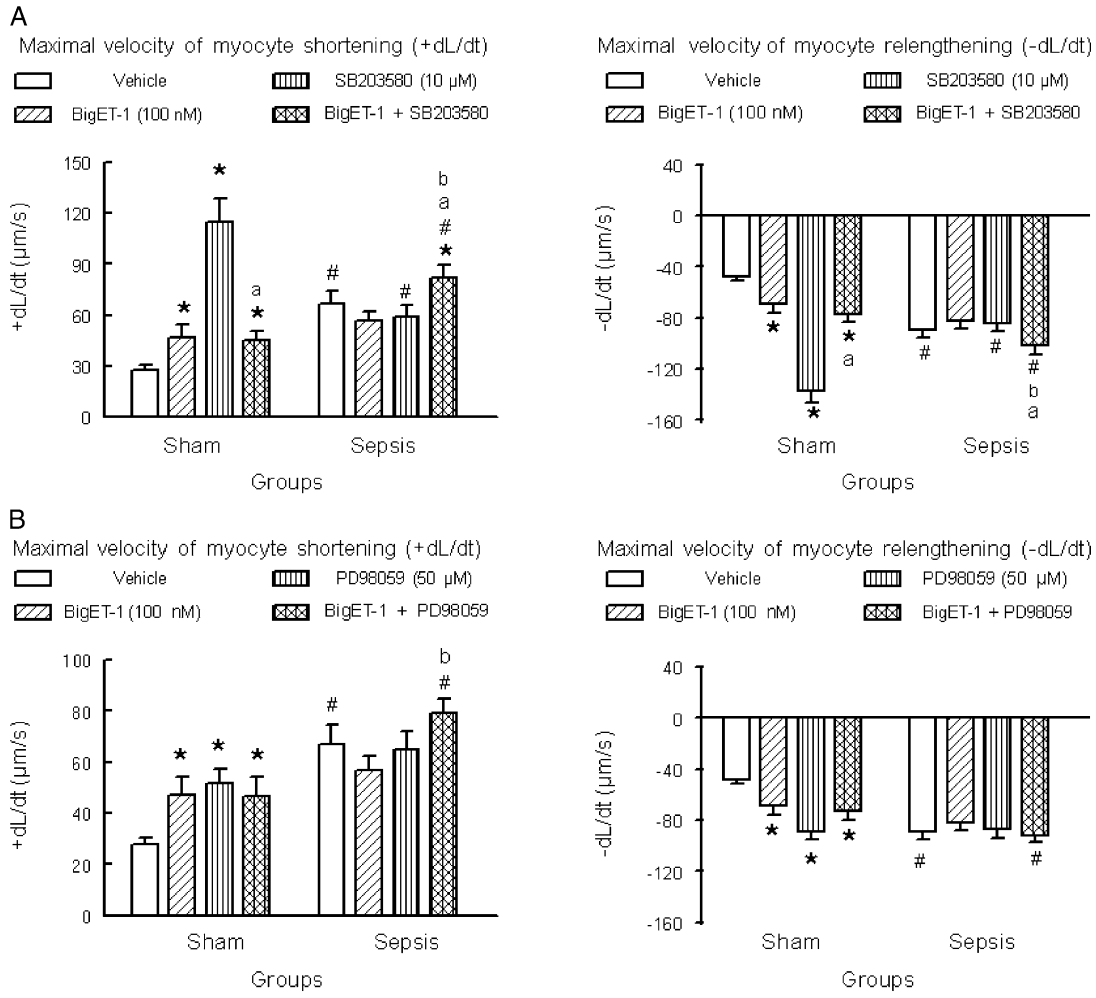

Fig. 6. Effect of inhibitors (A) SB203580 (10 μM) and (B) PD98059 (50 μM) on bigET-1 (100 nM) induced effect on (1) maximal velocity of myocyte shortening (+dL/dt) and (2) maximal velocity of myocyte relengthening (−dL/dt) in sham and sepsis groups. Data are represented as mean ± S.E.M., n=50 per data group, $^{*}P \leq 0.05$ as compared to vehicle treatment, $^{\#}P \leq 0.05$ as compared to respective treatment in the sham group, $^{a}P \leq 0.05$ as compared to inhibitor treatment in the respective group, $^{b}P \leq 0.05$ as compared to bigET-1 (100 nM) treatment in the respective group.

treatment. Pretreatment with SB203580 followed by bigET-1 administration in sham ARVM produced a significant increase in p38-MAPK phosphorylation as compared to SB203580 treatment per se. In addition, SB203580 pretreatment significantly decreased bigET-1 induced p38-MAPK phosphorylation in sepsis group but not in sham group as compared to bigET-1 treatment per se (Fig. 7A).

PD98059 (50 μM) did not alter p38-MAPK phosphorylation in both sham and septic ARVM as compared to vehicle treatment in both the groups. Pretreatment of ARVM with PD98059 did not affect bigET-1 induced p38-MAPK phosphorylation in both sham and sepsis groups (Fig. 7B).

3.8. Effect of bigET-1 on the protein expression of ET_A and ET_B receptor in presence of SB203580 and PD98059

Sepsis significantly decreased ET_A receptor expression but increased ET_B receptor expression as compared to vehicle-treated sham group. BigET-1 did not alter ET_A expression but significantly increased ET_B receptor expression as compared to vehicle treatment in sham group. In sepsis group, bigET-1 produced a significant increase in ET_B receptor expression as compared to sham group (Fig. 8). BigET-1-induced elevated ET_B receptor expression in septic ARVM was also significant as compared to the respective sham group. Pretreatment with SB203580 (10 μM) followed by bigET-1 did not alter ET_A receptor expression in both sham and sepsis groups. Pretreatment with SB203580 significantly reduced ET_B receptor expression as compared to vehicle and bigET-1 treatment per se in the sepsis group. PD98059 (50 μM) pretreatment significantly increased ET_B receptor expression as compared to sham group, but did not alter ET_A receptor expression in both sham and sepsis groups (Fig. 8).

4. Discussion

Sepsis produces myocardial dysfunction in both humans and animal models [2–4]. Earlier, we have demonstrated

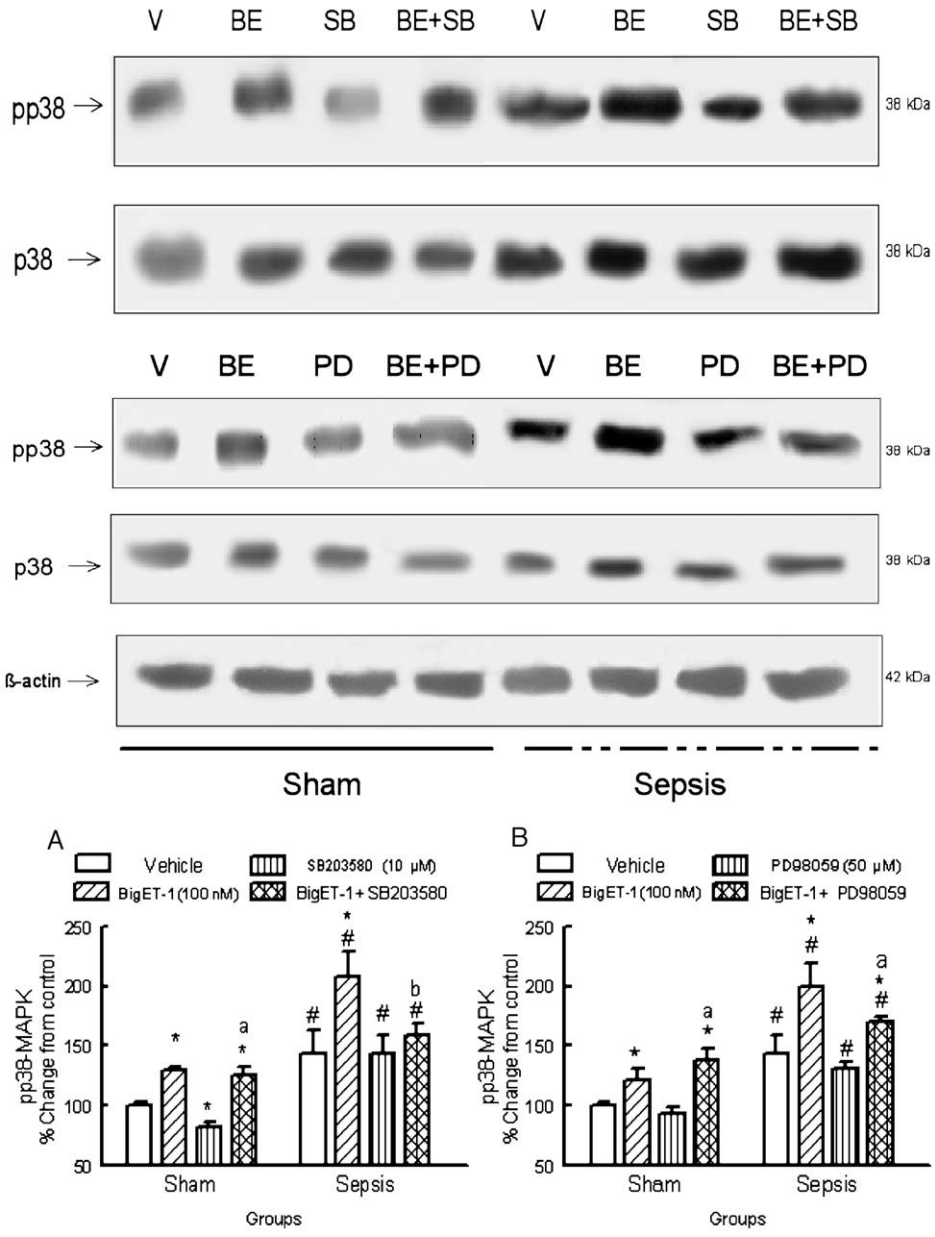

Fig. 7. Effect of inhibitors (A) SB203580 (10 μM) and (B) PD98059 (50 μM) on the protein expression of phosphorylated and total p38-MAPK in ARVM obtained from sham and sepsis groups in absence and presence of bigET-1 (100 nM). The blot in each case is a representative of three independent experiments. β-actin protein expression depicts uniform loading in gel. Data are represented as mean ± S.E.M., $^{*}P \leq 0.05$ as compared to vehicle treatment, $^{\#}P \leq 0.05$ as compared to respective treatment in the sham group, $^{a}P \leq 0.05$ as compared to inhibitor treatment in the respective group, $^{b}P \leq 0.05$ as compared to bigET-1 (100 nM) treatment in the respective group.

that chronic peritoneal sepsis in the rat depressed rates of left ventricular contraction and relaxation in an isolated heart preparation [3]. Similarly, depressed ARVM contractility was observed following sepsis [4]. Unlike sham, the mechanical properties of septic ARVM were found unresponsive to increase in extracellular calcium concentration (0.5–3 mM) [4]. Interestingly, in the present study, although septic ARVM exhibited depressed contractility at 3 h post-incubation in culture medium, the contractile properties were elevated at 24 h as evident by increase PS, +dL/dt and −dL/dt. It appears that the time difference of ARVMs in culture medium affects the contractility of septic ARVMs but not of sham group. Similar observations have been made by Ren et al. where myocytes isolated from spontaneously hypertensive rat (SHR) heart did not exhibit increased +dL/dt and −dL/dt which was in contrast to their earlier findings where they had demonstrated increased contractility by SHR myocytes [24]. The authors in that study attributed this dissimilarity to higher afterload in hypertension and the presence of non-myocyte factors, such as fibroblast or nerve terminals that may contribute to the differential contractile response. Septic ARVM in the present study exhibited

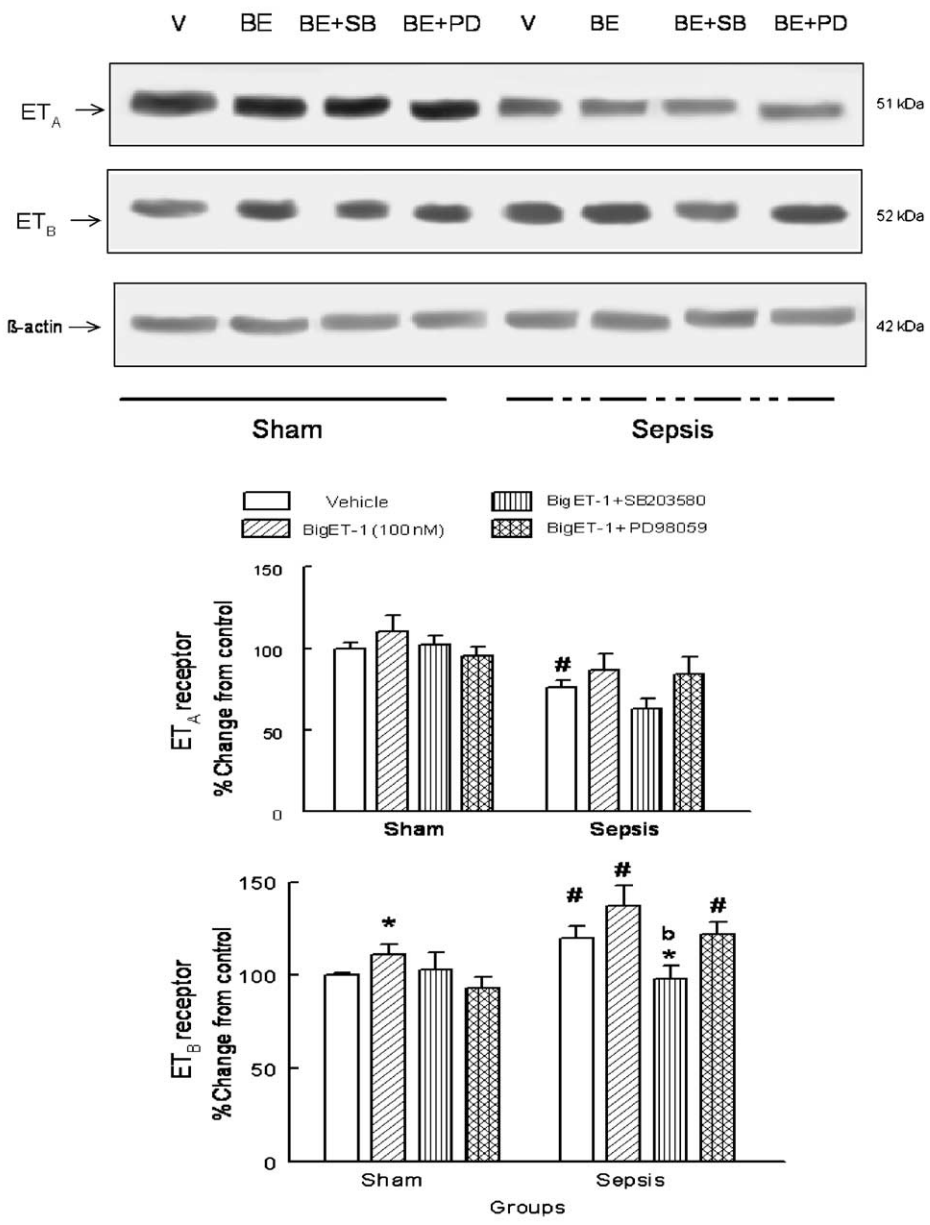

Fig. 8. Effect of SB203580 (10 μM) and PD98059 (50 μM) on the protein expression of ET_A and ET_B receptors in ARVM obtained from sham and sepsis groups in absence and presence of bigET-1 (100 nM). The blot in each case is a representative of three independent experiments. β-actin protein expression depicts uniform loading in gel. Data are represented as mean ± S.E.M., $*P \leq 0.05$ as compared to vehicle treatment, $^{\#}P \leq 0.05$ as compared to respective treatment in the sham group, $^{b}P \leq 0.05$ as compared to bigET-1 (100 nM) treatment in the respective group.

upregulated phosphorylation of both p38-MAPK and ERK1/2 post 24 h. It appears that septic ARVM in culture exhibited an increased contractility possibly due to prolonged activation of the MAPK signaling or via yet unknown active compensatory mechanism.

ET-1 acts as a systemic hormone and also as a local factor [25]. Besides exerting positive inotropic and chronotropic actions [19], ET-1 is a well-known mitogen and mediates load-induced hypertrophy in cultured neonatal ventricular myocytes [9]. Elevated ET-1 has been observed in various pathophysiological states like chronic heart failure (CHF) [26]. Wei et al. demonstrated that elevation of plasma ET-1 in severe CHF in humans principally represents elevation of the ET-1 precursor, bigET-1 [27]. In severe CHF, bigET-1 and ET-1 have been shown to represent 60% and 40% of the total immunoreactive plasma ET-1, respectively [28]. Concentration of ET-1 in both plasma and myocardium were found elevated during sepsis and endotoxemia [3,21,22]. However, the role of elevated ET-1 during sepsis and septic shock is not completely understood. During endotoxemia, it was observed that inhibition of ET-1 biosynthesis during early stage down-regulated p38-MAPK phosphorylation and nitric oxide synthase II (NOS 2) [21,22]. In the present study, exogenous

administration of ET-1 precursor, bigET-1, elevated the concentration of ET-1 in ARVM supernatants and produced hypertrophy both in sham and sepsis groups. In addition, bigET-1 (100 nM) elevated ET-1 by 22 pg/g (~3-fold) in sham group vs. 16 pg/g (~6-fold) in sepsis group. Both endogenous ET-1 biosynthesis and exogenous availability of bigET-1 may account for the observed increase of ET-1 concentration in both sham and sepsis groups. Surprisingly, septic ARVM themselves have less basal ET-1. We speculate that this decreased ET-1 concentration in septic ARVM could be due to depressed intrinsic ECE-1 activity, reduced preproET-1 mRNA or alterations in pretranscriptional regulation of ET-1 gene.

BigET-1 exerted a positive inotropic effect in sham ARVM and up to 3 h in septic ARVMs. However, septic ARVM did not exhibit any further alterations in PS, +dL/dt and −dL/dt following treatment of bigET-1 at 24 h post-treatment. This non-responsive effect of the otherwise positive inotrope bigET-1 could be due to several reasons. The first reason could be the activation of p38-MAPK that is stimulated by both sepsis (30% increase vs. sham) and bigET-1 (50% increase vs. vehicle-treated septic ARVM) as seen in the present study. The data suggest that sepsis-induced p38-MAPK phosphorylation is further potentiated by bigET-1 administration. However, in sham ARVM bigET-1 produced ~1.4-fold increase as compared to vehicle-treated sham ARVM. Chen et al. [29] have shown that p38-MAPK activation decreases force development in ARVM. Also Liao et al. demonstrated that a 3.4-fold increase in phosphorylation of p38-MAPK leads to 45% reduction in the extent of cell shortening [30]. We speculate that this additive increase in p38-MAPK activation following 24-h bigET-1 treatment in septic ARVM resulted in a decrease in contractility that may contribute to prevent the positive inotropic effect of bigET-1 via ET_A receptors during sepsis. It is further supported by the data that demonstrated that septic ARVM exhibited downregulated ET_A receptor expression. We suggest that the non-responsive effect of chronic bigET-1 during sepsis could be due to downregulated ET_A and upregulated ET_B receptors as seen in the present study. This upregulation of ET_B receptors via mature ET-1 may cause an excessive NO generation via stimulation of inducible NOS (NOS2) [6,21,25]. In another study, we have observed that bigET-1 in septic ARVM causes increase in iNOS and NO byproducts that may contribute to an increase in NO [31]. This is supported by the evidence that NO attenuates the direct effects of ET-1 in isolated rat hearts and decreases the ability of the peptide to increase intracellular levels of calcium in freshly isolated ARVM [32]. Furthermore, NO has been shown to mask the basal vasoconstrictor effect of ET-1 in normal human subjects [33]. We suggest that ET-1 induced increased NO could be one of the mechanisms responsible for non-responsiveness of bigET-1 on septic ARVM contractility. However, further studies will be required to support this speculation.

4.1. Role of p38-MAPK in regulating ARVM contractility during sepsis

The present study provides evidence for the involvement of MAPK cascade in ARVM contractility during sepsis. The MAPK family is an important family of kinases that transmits extracellular stimuli to various cellular functions including proliferation and protein synthesis [13]. MAPK(s), particularly p38-MAPK, have been implicated in cellular response to various stresses, such as exposure to UV, osmotic shock, mechanical or chemical stress and heat shock [13]. Both endotoxemia and sepsis produce an upregulation of myocardial p38-MAPK phosphorylation [17,21,22]. Similar observations were made by Song et al. where they demonstrated that p38-MAPK inhibits immune suppression in splenocytes in a CLP mouse model of polymicrobial sepsis [34,35]. Although they did not detect any JNK signal from lymphocytes, they could specifically block the cytokine IL-10 release by p38-MAPK inhibitor SB203580. The data obtained in the present study indicated that SB203580 increased +dL/dt and −dL/dt in a dose-dependent fashion in sham ARVM. SB203580 that inhibited p38-MAPK phosphorylation also increased PS by ~2-fold in sham group. These data suggest that p38-MAPK exerts a pronounced negative inotropic effect in sham ARVM. The findings suggest the involvement of p38-MAPK as a negative inotrope in the regulation of ARVM contractility. Similar observations were made by Liao et al. where they demonstrated a negative inotropic effect of p38-MAPK and its inhibitory effect on β-adrenergic receptor mediated positive contractile response in ARVM [30]. SB203580 increased the contractile function properties of septic ARVM suggesting that p38-MAPK has an opposite effect on ARVM contractility in sepsis vs. sham. This was further supported by the observation that p38-MAPK inhibition followed by bigET-1 elevated ARVM contractile function in sepsis group but not in sham. It appears that bigET-1 induced p38-MAPK modulation contributes differentially in the regulation of sham and septic ARVM contractility.

To dissect the role of ERK1/2 in bigET-1-induced ARVM contractile response, PD98059, an ERK pathway inhibitor, was used in the study. It has been demonstrated that PD98059 is highly specific for MEK1/2, the upstream activator of ERK1/2 with no effect on other kinases including p38-MAPK and JNK [36]. We did not observe any effect of PD98059 (50 μM) on p38-MAPK phosphorylation. PD98059 produced an increase in the contractile amplitude of ARVM in sham group although not in a dose-dependent fashion suggesting that ERK1/2, similar to p38-MAPK, mediates a negative inotropic effect on sham ARVM. Although PD98059 pretreatment followed by bigET-1 did not affect PS and −dL/dt it stimulated +dL/dt in septic ARVM. These data suggest that the ERK1/2 pathway of the MAPK cascade is involved in part along

with p38-MAPK in bigET-1 induced ARVM contractility during sepsis.

ET-1 has been shown to activate p38-MAPK in neonatal rat cardiac myocytes [12] and ARVM [37]. In the present study, bigET-1 upregulated p38-MAPK phosphorylation in sham and septic ARVM at early (5, 15 and 30 min post-treatment) and late (24 h post-bigET-1 treatment) time points. We also observed that ERK1/2 phosphorylation was not affect by bigET-1 treatment. Similar observations were made by Markou et al. where they showed that ERK1/2 activation by ET-1 is transient that reached maximal at 2–5 min while p38-MAPK phosphorylation was delayed compared with that of ERK1/2 following ET-1 treatment in ARVM [37]. Another study by Xiao et al. [38] demonstrated noradrenaline-stimulated ERK1/2 activation that persisted for 48 h in ARVM. In the present study, ERK1/2 activation was more intense in septic ARVM as compared to sham. Prolonged ERK1/2 activity in cardiac myocytes has also been reported in response to oxidative stress [39]. Barron et al. also have shown the occurrence of a second peak of ERK activation following an initial early peak of ERK activity on phenylephrine treatment in neonatal rat ventricular myocytes [40]. This is consistent with our findings that demonstrate ERK1/2 phosphorylation at 24 h in both sham and septic ARVM.

SB203580 attenuated p38-MAPK phosphorylation in sham ARVM as was reported by other research groups [30]. However, we did not observe depression of p38-MAPK phosphorylation by SB203580 in septic ARVM possibly due to the preexisting increased p38-MAPK phosphorylation during sepsis. In another study, Mockridge et al. showed that SB203580 was unable to block p38-MAPK phosphorylation but still prevented the downstream kinase effect of p38-MAPK on ATF2 phosphorylation [41]. It appears that MAPK mechanisms that differ in sepsis and sham ARVM may be responsible for the inability of bigET-1 to produce any positive inotropic effect in sepsis. In the present study, pretreatment with SB203580 but not PD98059 altered bigET-1 induced p38-MAPK phosphorylation in septic ARVM. These data suggest that primarily p38-MAPK is involved in bigET-1-induced response on ARVM contractility during sepsis. In addition, pretreatment with SB203580 reversed bigET-1 induced upregulation of ET_B receptor expression. These data reveal that p38-MAPK is involved in bigET-1 induced effects in ARVMs during sepsis. However, further studies are warranted to elucidate the upstream and downstream effectors of bigET-1-induced p38-MAPK activation in ARVMs during sepsis.

The results of the present study demonstrate that bigET-1 treatment in ARVM elevated ET-1 concentration that correlated with upregulation of ET_B receptors and p38-MAPK phosphorylation in both sham and sepsis groups. Although bigET-1 produced ARVM hypertrophy and increased the contractility of sham ARVM, it did not affect the septic ARVM contractile function. We concluded that a bigET-1-induced non-responsive effect on septic ARVM contractile function could be due to upregulation of p38-MAPK phosphorylation and upregulated ET_B receptor expression.

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